A Gel Filtration Method for Determining Total Iron-Binding Capacity in Serum

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A method is described for determining the total iron-binding capacity of serum by gel filtration.
Excess iron is added to the serum to saturate the transferrin. The free iron is bound as ferroin in a complex with o-phenanthroline. Fractionation is then performed on a Sephadex G-25 column, and the iron in the serum effluent is determined with dipyridyl.
The method has the advantage that it utilizes the whole specimen thus requiring smaller serum samples. Furthermore it incorporates phenol as a deproteinising agent producing optically clear supernatants.

Es wird eine Methode für die Bestimmung der Gesamt-Eisen-Bindungskapazität des Serums durch Gelfiltration beschrieben.
Zu Serum wird Eisen in Überschuß hinzugefügt, um das Transferrin zu sättigen. Das freie Eisen wird als Ferroin in einem Komplex mit o-Phenanthrolin gebunden. Die Fraktionierung wird dann an einer Sephadex-G-25-Säule durchgeführt und das Eisen im ausfließenden Serum mit Dipyridyl bestimmt.
Die Methode hat den Vorteil, daß sie die gesamte Probe verwertet und daher kleinere Serumproben benötigt. Ferner wird Phenol zum Enteiweißen benutzt; das ergibt klare Überstände.

It is usual to determine and indicate the transferrin in serum indirectly by the maximum amount of iron which it can bind, the so-called total iron-binding capacity (TIBC).

As the degree of saturation of the transferrin is normally only about 0.30, the first stage of a TIBC-determination consists of adding an excess of iron to total saturation, then removing the excess non-protein-bound iron prior to the final serum iron determination.

Among methods for removing the free iron are ion exchange (1), adsorption on magnesium carbonate (2), or binding in a complex (ferroin) with o-phenanthroline, which can then either be adsorbed on activated charcoal or precipitated by trichloroacetic acid (3).

Considering the molecular size it should be possible to separate protein-bound iron and electrolyte iron by means of a column of the highly cross-linked dextran, Sephadex G-25, using a bed volume 5—10 times the sample volume (4).

This principle was employed by Barber, Dempster and Anderson (5) in determining the unsaturated iron binding capacity (UIBC = TIBC ÷ actual Fe): $^{59}FeCl_3$-enriched serum was fractionated on a Sephadex column and the radioactivity in the eluate recorded continuously.

Contrary to expectation the elution diagrams from this study show that the free iron can only by separated sharply from the protein fraction by using the lower cross-linked dextran Sephadex G-50, and 4—5 times the theoretical hight of column. As a result the sample volume is diluted approximately 40 times. This is of no great significance in the tracer method, but it does have the result that the procedure is incompatible with a colorimetric determination of the iron. I have confirmed the elution pattern obtained by Barber et al. (5) (fig. 1).

A probable explanation of the discrepancy between theory and practice is that at the pH employed, 7.5, the iron behaves as a colloid, and is thus partly excluded from the gel. On the other hand, if the free iron is bound in a complex with o-phenanthroline by Laurell's method (3), the sharp separation demanded by the theory is realized (fig. 2). The iron in the serum effluent can then be determined colorimetrically.

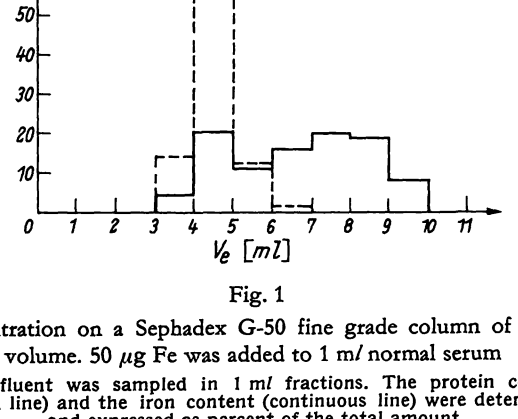

Fig. 1

Gel filtration on a Sephadex G-50 fine grade column of 10 ml volume. 50 µg Fe was added to 1 ml normal serum

The effluent was sampled in 1 ml fractions. The protein content (dotted line) and the iron content (continuous line) were determined and expressed as percent of the total amount
Flow rate 5—6 ml per hour
Tris-citrate buffer pH 7.5

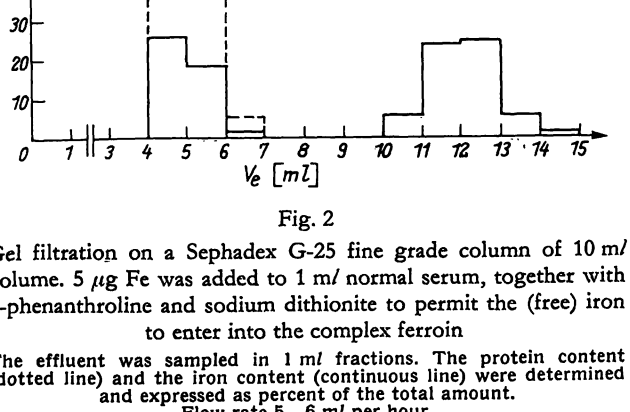

Fig. 2

Gel filtration on a Sephadex G-25 fine grade column of 10 ml volume. 5 µg Fe was added to 1 ml normal serum, together with o-phenanthroline and sodium dithionite to permit the (free) iron to enter into the complex ferroin

The effluent was sampled in 1 ml fractions. The protein content (dotted line) and the iron content (continuous line) were determined and expressed as percent of the total amount.
Flow rate 5—6 ml per hour
Tris-citrate buffer pH 7.5

Methods and materials

The serum is enriched with iron in the presence of sodium dithionite. o-phenanthroline is then added, whereby the free iron forms ferroin, $(C_{12}H_8N_2)_3Fe^{++}$ (3).
This mixture is now fractionated by gel filtration. The iron content of the serum effluent is then determined with dipyridyl according to the method of RAMSAY (2) modified by the addition of phenol, which results in complete deproteinisation (6).

Apparatus

Columns, divided by a constriction of 3 mm inner diameter into a lower chamber, 10 × 110 mm fitted with a capillary outlet with plastic tube, and an upper chamber, 15 × 150 mm (7). The upper chamber is modified by means of a Quickfit air inlet adaptor to maintain a constant hydrostatic pressure.

Plastic tubes, 70 × 11 mm.
Plastic tubes, 100 × 16 mm (Makrolon) + screw cap } (Nunc, Roskilde, Denmark)

Constriction pipettes, 50 µl and 1000 µl.
Pipettes, 6.00 ml and 7.00 ml.
Beckman DU spektrophotometer with 100 mm cell compartment for use with 50 mm half micro cuvettes.

Reagents

All chemicals are analytical grade.
Tris solution 25 mM: Tris(hydroxymethyl)aminomethane, 3.0 g/l.

Tris-citrate buffer pH 7.5: Tris(hydroxymethyl)aminomethane, 6.06 g, citric acid (1 H_2O), 2.85 g, sodium chloride 5.80 g, dissolved in deionised water to give a final volume of 1 liter.

Iron solution 1.8 mM: Iron(III)-chloride (6 H_2O), 485 mg, 5 ml 1M hydrochloric acid, and deionised water to give a final volume of 1 liter.

o-phenanthroline solution 16.8 mM: o-phenanthroline chloride (H_2O) 39 mg, dissolved in 10 ml tris solution 25 mM. This corresponds to a saturated solution of phenanthroline base.

Sodium dithionite solution 115 mM: Sodium dithionite 20 mg, dissolved in 1 ml Tris solution 25 mM prepared immidiately before use.

Dipyridyl-phenol solution, 19.2 mM and 530 mM respectively: 2,2-dipyridyl 3.0 g, acetic acid 90 ml, phenol 50 g, dissolved in deionised water to give a final volume of 1 liter.

Sodium sulfite solution 300 mM: Sodium sulfite 3.8 g, dissolved in deionised water to give a final volume of 100 ml. Prepared daily.

Iron stock solution 1.79 mM: Iron wire 100 mg, dissolved in 50 ml concentrated hydrochloric acid and brought to 1 liter with deionised water.

Iron standard 53.7 µM: Iron stock solution 30.00 ml, brought to 1 liter with deionised water.
Sephadex G-25, fine.

Packing the column

The column is placed vertically in a stand, and the outlet covered with a perlon net, Monodur 45, corresponding to a mesh of 45 µm. The lower chamber is then filled with the eluent buffer, and the upper chamber filled with a suspension of Sephadex G-25 which has been allowed to swell in buffer for at least 24 hours. When the lower chamber is full, the superfluous Sephadex is removed and the column then stabilized by allowing buffer to run through (a volume 4—5 times the bed volume). If the column settles during use, it is filled up again with the Sephadex suspension to the middle of the constriction.

Procedure

Serum sample

1000 µl of serum, 50 µl of the iron solution (~ 5 µg iron) and 50 µl of the sodium dithionite solution (~ 1 mg dithionite) are pipetted into a disposable plastic tube, 70 × 11 mm. After standing for 10 min., 50 µl of the o-phenanthroline solution is added, then the mixture is allowed to stand another 10 minutes. The mixture is then transferred to the Sephadex column by means of a 1000 µl constriction pipette. While the sample is entering the gel bed, the effluent is allowed to run to waste. 7.00 ml tris-citrate buffer pH 7.5 is then pipetted into the upper chamber which is thereafter fitted with an air inlet adaptor. The tip of this is placed about 1 cm above the constriction.
The effluent is collected in a makrolon tube, 110 × 16 mm, the flow being regulated to 5—6 ml per hour by raising and lowering the tip of the outlet tube.
A volume of 1000 µl dipyridyl-phenol solution and 1000 µl sodium sulphite solution are added to the effluent, and the makrolon tube is then fitted with a closely fitting screw cap and put in a boiling water bath for five minutes. After thorough cooling in running tap water, the tube is then centrifuged at 2000 *g* for 20 min.

Standard

1000 µl of the iron standard solution + 6.00 ml tris-citrate buffer pH 7.5 receives the same treatment as the serum effluent.

Reagent blank

7.00 ml tris-citratebuffer pH 7.5 receives the same treatment as the serum effluent.
Readings are taken on a Beckman DU spectrofotometer at 522 nm in 50 mm half micro cuvettes.
The standard and the supernatant from the samples are read against the reagent blank, giving extinction E_{st} and E_p respectively.

Calculation

$$\text{TIBC} = \frac{E_p}{E_{st}} \cdot 53{,}7\ \mu\text{mol/l} \left(\frac{E_p}{E_{st}} \cdot 3\ \text{mg/l} \right).$$

After elution of the protein from the column the upper chamber is filled with approximately 15 ml tris-citrate buffer. When this has almost all run through, and there is fluid at a height of about 0.5 cm, the outlet tube is closed with a little plastic rod and the column can remain in this state until the next analysis.

Results

Normal value

Analysing the readings in 27 donors (16 men and 11 women) a mean value of 55.6 µmol/l (3.10 mg/l) was found with a standard deviation of 7.3 µmol/l (0.41 mg/l).
This agrees fairly well with the result obtained by LAURELL (3) who arrived at a mean value of 3.15 mg/l, and a standard deviation of 0.33 mg/l in a total of 69 men and 31 women. LAURELL found no grounds for assuming any sex difference.

Precision

The precision of the method is illustrated by calculating the standard deviation from the 27 double determinations using the formula $\sigma = \sqrt{\frac{\Sigma d^2}{2 \cdot n}}$, where d is the difference between the respective double values, and n is the number of double determinations. $\sigma = 1.8$ µmol/l (0.10 mg/l). This corresponds closely to the value found by the ion exchange method (8).

Discussion

In the determination of TIBC it is necessary to operate at a pH > 7.2 in order to avoid dissociation of the iron-transferrin complex (3). So as not to fall lower than the

critical pH on adding the acidic dithionite, LAURELL released carbon dioxide from the serum by deep freezing for 24 hours, followed by thawing. In the present investigation the procedure followed was to buffer the reagent to pH approximately 7.5 by adding Tris, so that even fresh serum could be used without any risk.

The makrolon tubes supplied are packed in dust-tight containers and can be used immediately so that time-consuming cleaning of glass ware is avoided.
The use of phenol results in a complete deproteinisation during the heating and the supernatant is optically clear.

References

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Qualitative Veränderungen verschiedener jodierter phenolischer Aminosäuren im Laufe einer alkoholischen Extraktion aus wäßrigen Lösungen

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Es werden die chemischen Veränderungen, die einige jodierte phenolische Aminosäuren im Verlaufe einer Extraktion aus wäßrigen Lösungen erfahren, untersucht. Die durchgeführten chemischen und dünnschichtchromatographischen Kontrollen zeigen, daß die prozentualen Verluste der Ausgangssubstanzen meistens relativ geringfügig sind. Die im Verlauf der Extraktion neugebildeten Verbindungen lassen sich, mit nur einer Ausnahme, als jodierte phenolische Aminosäuren identifizieren.

The qualitative changes which occur in a number of iodinated phenolic amino acids during alcoholic extraction from aqueous solutions, were investigated.
Thin-layer chromatographic controls showed that in most cases the decomposition of the starting materials is relatively small. The new compounds formed during the extraction can be classified, with one exception, as iodinated phenolic amino acids.

Aufgrund früherer Untersuchungen über die Verteilungskoeffizienten verschiedener jodierter phenolischer Aminosäuren in einer Reihe wäßrig-organischer Zweiphasen-Systeme (1) wurde eine Methode entwickelt, diese Substanzen aus dem Serum zu gewinnen. Die hohe Ausbeute ermöglicht nach weitergehender Reinigung des Extraktes in Verbindung mit einem jüngst beschriebenen dünnschichtchromatographischen System (2) die Trennung und Identifikation zirkulierender Schilddrüsenhormone und Derivate (3, 4).
Die Frage der eventuellen Zersetzung der jodierten Substanzen im Verlauf der Extraktion wird hier mittels reiner wäßriger Lösungen verschiedener Jodtyrosine und Jodthyronine, die der Extraktion unterworfen wurden, näher untersucht. Die Kontrollen wurden mit Hilfe der Dünnschichtchromatographie durchgeführt. Die Anwendung einer relativ einfachen Technik — auch hier beschrieben — gestattet die Auswertung der Intensität der vorhandenen Flecken auf den Chromatogrammen durch Extinktionsmessung.

Methodik

Als Versuchssubstanzen wurden folgende jodierte phenolische Aminosäuren verwendet: 3-Monojod- und 3,5-Dijod-L-Tyrosin, 3-Monojod-, 3,5-Dijod-, 3,3′,5-Trijod- und 3,3′,5,5′-Tetrajod-DL-thyronin (MIT, DIT, T_1, T_2, T_3, T_4). Diese Substanzen wurden mit 0,05N NaOH (0,5 mg pro ml) gelöst und getrennt bearbeitet.

Extraktionsverfahren

Die Extraktion wurde folgendermaßen durchgeführt: 6 ml von jeder der angesetzten Lösungen (gleich 3 mg Versuchssubstanz) wurden in Zentrifugenröhrchen mit Schliff (etwa 65 ml Fassungsvermögen) mit 0,5 ml 1M Propylthiouracillösung versetzt, mit einigen Tropfen konz. Essigsäure auf pH <5 eingestellt und 5 Min. mit 15 ml Methanol am Rückflußkühler gekocht.
Die wäßrig-alkoholische Phase wird nun mit 2 g einer Mischung Natriumsulfat-Aktivkohle 200:1 geschüttelt, zentrifugiert und nach Abtrennung des Rückstandes in ein anderes Schliffröhrchen überführt und am Rotationsverdampfer bei Unterdruck und 55—60° eingeengt. Der Unterdruck wurde durch eine Wasserstrahlpumpe erzeugt. Der trockene Rückstand wird mit 3 ml 0,05N NaOH aufgenommen und in ein Zentrifugenröhrchen mit einem Fassungsvermögen von etwa 45 ml gebracht. Das vorherige Röhrchen wird mit 3 ml 0,05N NaOH nachgespült und dieser Anteil mit dem anderen gemischt. Die wäßrige Lösung wird nach Kontrolle mit